IDENTIFICATION OF SMOOTH MUSCLE GAMMA ACTIN IN PROSTATE EPITHELIA

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Identification of Smooth Muscle Gamma Actin in Prostate Epithelia. (May 2014)

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Prostate cancer is the second most common form of cancer in American men [1]. Though broad treatments are available, these often have deleterious effects on the patient. One approach which may result in a more tailored treatment for prostate cancer resides in the understanding of the expression of smooth muscle gamma actin (SMGA) in the physiology of cancerous prostate epithelial cells. It is important to focus on SMGA due to its increased expression in the prostate epithelia during cancer progression. Previous research indicates that SMGA protein is present in the prostate epithelia, but as of yet, it has not been detected by traditional methods. The goal of this study is to determine the discrepancy between SMGA protein found in the prostate epithelia from that found in the prostate smooth muscle.

In order to demonstrate SMGA protein is in fact present in prostate epithelia, we subjected lysates derived from a prostate cancer cell line, PC-3, to Western blot analysis, immunoprecipitation, and mass spectroscopy. We used monoclonal muscle actin isoform-specific antibody, HUC1-1, to determine that these isoforms were present in lysate samples. As experimental controls, actin purified from chicken gizzard smooth muscle cells was run in parallel to the lysate on the same gel. We next used HUC1-1 in immunoprecipitation experiments to precipitate actin from the lysate. We found that its band matched to the control of purified actin which migrated at 43 kDa on the gel system. The band from the precipitated actin was then cut from the gel and subjected to mass spectroscopy

analysis for sequencing. These experiments identified multiple peptides that are diagnostic of SMGA. However, we did not detect the amino terminus of the prostate epithelial specific-SMGA.

Suggested studies would be: 1) to sequence the amino acids at the N-terminus using Edman degradation and compare the sequenced protein to SMGA found in mature smooth muscle; and in the long term, 2) synthesize an antibody specific to prostate epithelial specific-SMGA to determine its function in cancerous prostate.

DEDICATION

I dedicate this thesis to Patrick Rock. Without him, the formatting of this paper would not have been possible. Thank you for introducing me to LaTeX and having the patience to fix all my errors. I appreciate you more than you will ever know. I wish you the best in your future, whether it be in medicine, computer science, or as a jazz musician.

ACKNOWLEDGMENTS

I would like to thank Dr. Warren Zimmer for providing me with the opportunity to work in his lab and introducing me to the field of research. His patience with me over the past two years encouraged me to persevere with this project. Thank you for having confidence in me, even when I didn't.

I would also like to acknowledge the Louis Stokes Alliance for Minority Participation (LSAMP). Their dedication to undergraduate research encouraged me to experience research early on in my undergraduate career and more importantly, to continue each year.

NOMENCLATURE

SMGA	Smooth Muscle Gamma Actin
kDA	Kilodaltons
MET	Mesenchymal-epithelial transition
EMT	Epithelial-mesenchymal transition
mRNA	Mitochondrial Ribonucleic Acid
G-Actin	Globular Actin
F-Actin	Filamentous Actin
FBS	Fetal Bovine Serum
RIPA	Radioimmunoprecipiation assay
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TEMED	Tetramethylethylenediamine
TBST	Tris Buffered Saline Tween-20
PBST	Phosphate Buffered Saline Tween-20
PTM	Post-Translational Modifications

CHAPTER I

INTRODUCTION

Manifestation of Prostate Cancer

In males, the second leading cause of death by cancer originates in the prostate [1]. Though broad treatments are available, these often have deleterious effects on the patient. One approach which may result in a more tailored treatment for prostate cancer resides in the understanding of the expression of smooth muscle gamma actin (SMGA) in the physiology of cancerous prostate epithelial cells.

Anatomy and Physiology of the Prostate

The prostate is an exocrine gland of the male reproductive system. Located inferior to the bladder, it surrounds the urethra and its primary role is to secrete nutrients vital to the composition of seminal fluid. The human prostate undergoes growth at three stages during the course of the male's life: 1) prior to birth, 2) during puberty and 3) with the advancement of age. The stimulation of its growth is dictated by the presence of testosterone and other androgens [2]. The prostate is divided anatomically into three major areas: the 1) transitional, 2) central, and 3) peripheral zone [3]. Covering the most surface area is the peripheral zone, where 70% of adenocarcinomas are found.

Prostate Development

The prostate arises from endodermal tissue of the urogenital sinus during late embryogenesis. The adult prostate arises from contributions from the mesenchyme, urethra, and Wolffian ducts [4]. During development, it relies on the presence of androgens in order to differentiate [5]. This process is dependent on the feedback between mesenchyme, which will become the smooth muscle, and the epithelium. Androgens act on receptors in the urogential mesenchyme, to induce differen-

tiation into epithelial subtypes through a mesenchymal-epithelial transition (MET). However, both prostatic smooth muscle and epithelia are necessary for each others development [6]. While MET occurs during normal development from the urogential mesenchyme, the opposite process (EMT) may abnormally take place. During epithelial-mesenchymal transformation (EMT), a normal, polarized epithelial cell regresses back into a mesenchymal cell; this process is usually witnessed at the initiation of cancer progression [7].

Molecular Mechanisms in the Prostate

One of the key genes in prostate development is the androgen-regulated homeobox Nkx3.1. The homeobox gene superfamily directs the formation of many body structures during early embryonic development. These genes contain highly conserved DNA sequences which provide instructions for making homeodomain proteins, which act as transcription factors. In mouse models, Nkx3.1 has demonstrated its significance for prostate epithelial differentiation and stem cell function [5]. In cancerous prostate, Nkx3.1 expression has been found to decline as the cancer progresses [8]. Though Nkx3.1 is primarily expressed in adult prostate epithelia, it regulates many genes; among them is smooth muscle gamma actin (SMGA) [9].

SMGA protein, typically found in smooth muscle cells, has not been detected in the epithelia by the conventional method of anti-SMGA B4 clone. However, previous research has shown that SMGA mRNA transcripts are expressed in prostate epithelium [10]. This finding supports the theory that the protein is present in the cell, but may be slightly altered relative to normal SMGA in smooth muscle cells and thus, incognito to previous locating techniques using B4 antibody. This hypothesis has been proven correct by experiments using the anti-actin HUC1-1 clone.

Anti-muscle actin HUC1-1 antibody recognizes all four muscle actins: α-skeletal, α-cardiac, α-smooth, and SMGA by recognizing a consistent epitope in the middle of the actin sequence. While the SMGA-specific B4 antibody is unable to detect SMGA protein in prostate epithelia, the less

specific anti-muscle actin HUC1-1 is able to detect the presence of actin by Western blotting [11]. SMGA-specific B4 antibody recognizes SMGA at its N-terminus; one reason the B4 clone might be unable to recognize prostate epithelial-specific SMGA is due to an alteration at its N-terminus. Using immunohistochemistry with HUC1-1 clone, it has also been found that the immunoreactivity of HUC1-1 increases in more aggressive cancerous prostate epithelia [11].

Overview of Actin

Actin is one of the most highly conserved proteins between species. G-actin is characterized by a molecular weight of 42 kDa in both muscle and non-muscle cells. These G-actin monomers bind together to form the twisted helical chains of F-actin. The two ends of F-actin have different polarities which allow one end to lengthen and the other to shorten under necessary circumstances.

Types of Actin

Actins have been categorized by two different types: muscle or cytoplasmic. While muscle actin is usually located in striated and smooth muscle for contraction, cytoplasmic actin is more commonly found in non-muscle cells and contributes to cell regulation. Actin has been found to have distinct isoforms in vertebrates due to the differences in the amino acid sequence at the N-terminus. These isoforms have been classified as α, β, or γ, with α being the most acidic upon separation by two-dimensional gel electrophoresis [12].

Of the muscle actins, there are two forms of the striated and smooth muscle actins. α-skeletal muscle actin and α-cardiac muscle actin isoforms predominate in the skeletal and cardiac tissues, respectively [12]. Smooth muscle actins also have two forms: α-smooth muscle actin represents actin in vascular tissues and γ-smooth muscle actin constitutes actin in visceral smooth muscle. In mammals, cytoplasmic actins have two isoforms: β and γ, while more isoforms exist in birds and amphibians [13].

Function of Actin

The two types of actin each have their respective function. Muscle actins serve as framework for myosin during muscle contraction. They play a major role in the actinomyosin powerstroke pathway which allows for the basic principal of movement in physiology. Cytoplasmic actin has a more diverse range of functions including: cell motility, division, and cytoskeleton maintenance.

In summary, the role of SMGA in prostate epithelia has not yet been determined. However, its presence has been detected at the mRNA level and SMGA protein has been located by anti-muscle actin HUC1-1, though not by the conventional anti-SMGA B4. HUC1-1 immunoreactivity has been shown to increase as the cancer in the prostate epithelia progresses to become more aggressive, which suggests that the expression of this prostate epithelial specific SMGA also increases. This relationship indicates that this prostate epithelial specific-SMGA affects the normal cell metabolism. In this study, we plan to determine that the protein the anti-muscle actin HUC1-1 clone located is in fact SMGA.

CHAPTER II

METHODS

Cell Culture

The human prostate cancer cells used came from the PC-3 line and were purchased from ATTC (CRL#1435). The donor was a 62 year old Caucasian male and adenocarcinoma cells were in stage IV of cancerous progression. The cells were maintained in T-75 CellBind flasks and grown at 37^{o}C with 5% CO_2 with F-12 K media supplemented with FBS, Pen/Strep, and L-Glutamine. All cells used were passed less than five times in order to maintain cell character.

Cell Lysis

Cells were first washed with sterile 1x PBS, then chemically disrupted with RIPA buffer (50 mM Tris HCl at pH 7.4, 0.1% sodium deoxycholate, 150 mM sodium chloride, and 1% NP-40) supplemented with protease inhibitors. Cells were physically disturbed by scraping and then allowed to lyse on ice until viscous. Afterwards, cells were subjected to sonication in 30 second intervals until no longer viscous and then centrifuged at 12,000 rpm for 10 minutes to separate proteins from insoluble materials. The protein lysates were portioned into 1 mL aliquots and frozen at -20^{o}C until use.

Bradford Protein Assay

Protein concentrations were assessed using a flat bottom 96 well plate. Lysates were first diluted with water in 1:1, 1:10, and 1:50 ratios. The amount of protein standard γ-globulin ranged from 0.004 to .04 μg/μL in 4 μL intervals in duplicate to minimize error. The volume was then equalized with water to 160 μL. 10 μL RIPA buffer was diluted with 150 μL of water. The lysates were added to the plate in duplicate; 10 μL of each dilution was diluted with 150 μL of water. Finally,

40 μL of protein assay dye reagent (from Bio-Rad CAT #500-006) was added to each well with solution present to equalize the volume in each well to 200 μL. The absorbance was measured using a spectrophotometer at a wavelength of 595 nm. The data was analyzed using Multiskan Ascent Software Version 2.6. A standard curve of absorbance of the standards vs. their concentrations was determined. From this information, we were able to determine the amount of protein in each sample.

SDS-PAGE

Protein concentrations of lysates were analyzed on an SDS-PAGE gel. The discontinuous gel was made up of a lower 12% separating gel and a top 5.5% stacking gel. The 12% separating gel is prepared by using 6 mL of 30% Acryl/Bis, 3.75 mL of 1.5 M Tris at pH 8.8, 150 μL of 10% SDS, 5.03 mL of water; 7.5 μL of TEMED and 75 μL of 10% APS were added after about 1 hour of degassing. The stacking gel is made up from 1.98 mL of 30% Acryl/Bis, 3.78 mL of 0.5 M Tris at pH 6.8, 150 μL of 10% SDS, 9 mL of water; 15 μL of TEMED and 75 μL of 10% APS were added also after about 1 hour of degassing. The 12% separating gel was first pipetted into a Bio-Rad Criterion gel cassette and a negligent amount of water was used to remove any air bubbles. Once the 12% separating layer polymerized, the water layer on top was removed and the stacking gel then was pipetted on top of the 12% separating layer and let to polymerize with the comb inserted.

After the gel completely polymerized, the cassette was transferred to the gel box and filled with 1x running buffer (diluted from 1 L of 10x running buffer, which included 30.2 g Tris base, 10 g SDS, and 188 g glycine). The comb was carefully removed and the gel was loaded with: Novex Sharp Pre-Stained Protein Standard, purified actin (control), and lysate. The gel was run at 125 V for 2 hours. The cassette was then removed from the gel box and broken open to free the gel.

The gel was placed in a plastic box on a shaker and treated with Coomaisse blue for 2-3 hours to stain the protein contained within the gel. Afterwards, the Coomaisse blue was poured out and the

gel was treated twice with destain (10% acetic acid and 10% methanol in 1 L DDI H_2O) for 30 minutes, then let to sit overnight in destain. The gel was then vacuum dried at 70^{o}C for 2 hours and saved.

Western Blot

A Western blot confirmed that actin was present in the lysates. Separated protein was transferred overnight at 50 V to immonilon-P milipore membrane followed by 1 hour of blocking in 1x TBST containing 2% blocking agent (membrane blocking agent from GE Healthcare Lot #354916). We utilized HUC1-1 anti-actin from various suppliers (Santa Cruz Biotechnology Lot #J3108, MP Biomedical, and Thermo Scientific) We incubated the primary antibody mouse monoclonal IgG2a HUC1-1 in a 1:1,000 dilution overnight at 4^{o}C. Secondary antibody was used in 1:5,000 (anti-mouse IgG HRP from Promega) for 1 hour at room temperature followed by staining with ECL Western Blotting Analysis System by GE Healthcare. Subsequent chemiluminescence was detected using MultiGaugeV 3.0 software from a Fijifilm LAS-4000 camera.

Immunoprecipitation

In order to isolate the actin from the lysates, we performed an immunoprecipitation using HUC1-1 antibody. We utilized beads (Dynabeads Protein A from Novex by Life Technologies REF #10002D) and a magnet to separate the actin from the lysates and used purified actin as a control. A 50 μL quantity of beads was prepared for each sample.

To bind the antibody to the beads, we made a 1:3 dilution of HUC1-1 antibody in 1x PBST. We incubated the samples with rotation for 10 minutes to ensure the antibody bound to the beads.

The purified actin and lysates were prepared by thawing and subsequent resuspension. We made a 1:3 sample to PBST dilution. 15 μL of actin and each lysate was also saved to serve as the undiluted sample for future analysis on SDS-PAGE.

Next, we used the prepared bead-antibody complex to extract the actin from our samples. The lysates and purified actin were added to the prepared bead-antibody complex and incubated with rotation for 10 minutes to ensure that the HUC1-1 antibody bound the target antigen (actin). Tubes were placed on the magnet and the supernatant was saved for future analysis on SDS-PAGE. The bead-antibody-antigen complex was washed with 200 μL of PBST and two more washes were repeated to remove non-specific binding. The bead complex in the tubes was then removed and transferred to clean tubes to eliminate extraneous debris.

Finally, the actin of each sample was eluted from the bead complex. The tubes were placed on the magnet to remove the supernatants. 50 μL of 2x Laemmli sample buffer from Bio-Rad (CAT #161-0737) was added to the immunoprecipitated samples and 15 μL of 2x sample buffer was added to the undiluted samples. Prepared samples were heated for 10 minutes at 70^{o}C to unfold the proteins and then run on an SDS-PAGE gel at 125 V for 90 minutes.

Mass Spectroscopy

Mass spectroscopy was performed to analyze the peptides from the immunoprecipitated actin. The pulldown band from the lysate was cut from the gel and incubated in solution to release the peptides. The solution was then sent for mass spectroscopy analysis in order to determine the peptide sequence of the prostate epithelial-specific SMGA.

CHAPTER III

RESULTS

Bradford Protein Assay

This assay was performed to assess the amount of protein present in the samples. It is important to know the protein concentration so adequate dilutions can be considered for further experiments such as SDS-PAGE and Western blotting.

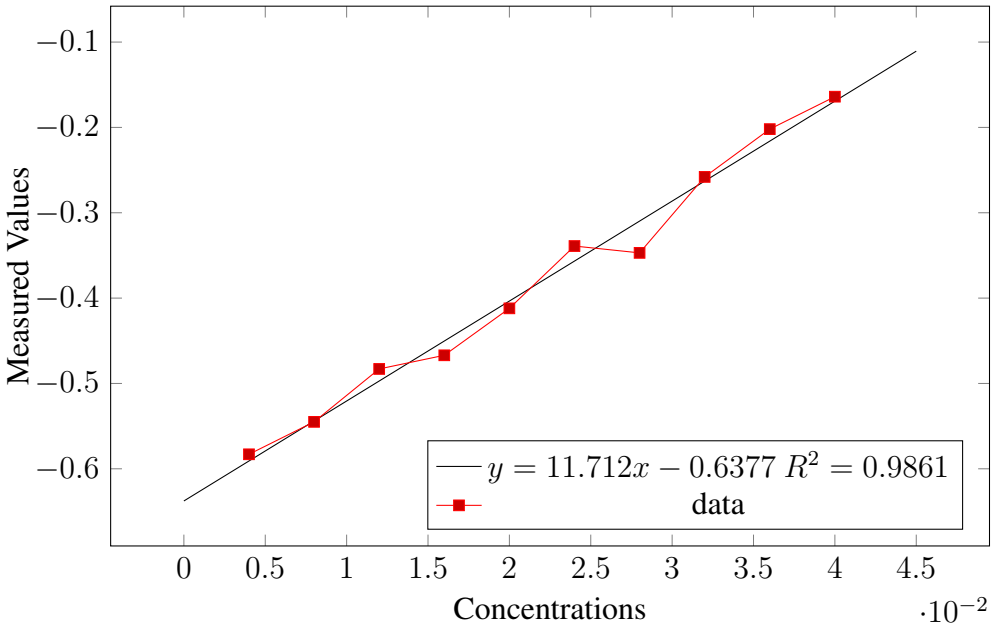


Fig. III.1.: Standard Plot of Protein Measured Values vs. Concentration

Fig. III.1 shows a graphical representation of the data and compares the concentrations of the proteins to line of best fit. The Multiskan Ascent Software analyzed the various dilutions of the control protein standard γ-globulin against the various concentrations of prepared lysates to generate the standard curve. The standard curve evaluates the relationship between the absorbance value of each sample and its concentration. The R^2 value indicates how well the data fits the line of best fit. While an R^2 = 1 indicates that the regression line matches the data exactly, an R^2 = 0 means

that the regression line found no correlation between the data.

From this assay, we determined that: 1) the R^2 = 0.9861, which means that the regression line found a strong correlation between our data points and that our data is reliable, 2) that we can determine the concentration of the proteins and future dilution factors with confidence, 3) the difference in concentration between our two lysates is not statistically significant and that both lysates will be treated with equal dilution factors.

SDS-PAGE

An SDS-PAGE verified that protein was present in both lysate samples and that both lysates had relatively similar concentrations. The even distribution of bands indicates that the protein in the lysate did not degrade. It is important to note that bands are visible near 42 kDa, the molecular weight of actin. This observation suggests that muscle actin is present in the prostate epithelial lysates and will be tested for detection by subsequent Western blotting.

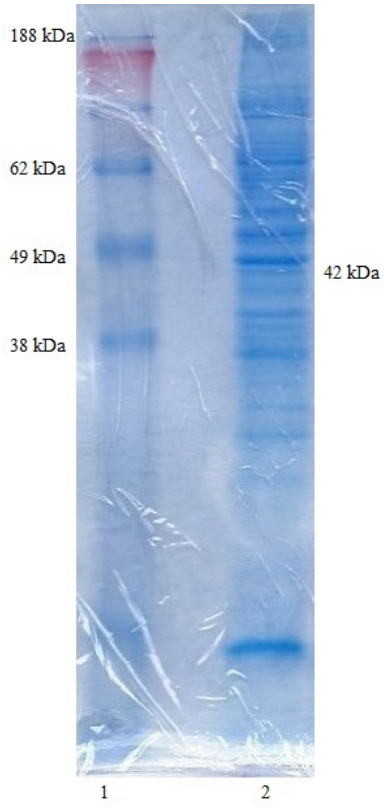
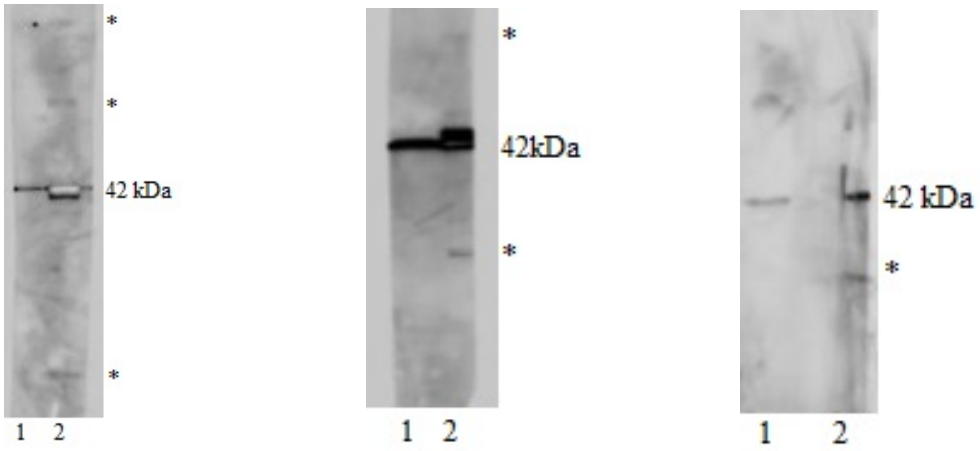


Fig. III.2.: Lane 1: Standard Marker, Lane 2: Lysate 1.

Western Blot

After confirming protein was present and intact in the lysate, we subsequently had to determine that a muscle actin was also present in the lysate of this non-muscle epithelial cell. Therefore, we performed a Western blot with anti-actin antibody from three different companies: MP Biomedical (Fig. III.3a), Santa Cruz Biotechnology (Fig. III.3b), and Thermo Scientific (Fig. III.3c).

Purified SMGA (control) and lysate were exposed to anti-muscle actin HUC1-1. The blots in Figures III.3, III.4, and III.5 were taken with a 30 second exposure time 24 hours after treatment with HUC1-1. All blots show that HUC1-1 bound to actin in both purified SMGA (control) and lysate as indicated by the black band at 42 kDa. Unspecific binding is denoted by the [*] asterisk.



(a) MP Biomedical. (b) Santa Cruz Biotechnology. (c) Thermo Scientific.

Fig. III.3.: Lane 1: Purified SMGA, Lane 2: Lysate. [*] denotes non-specific binding.

Fig. III.3a contains three bands of non-specific binding, but its band at 42 kDa is strong, which indicates that the antibody bound well to the actin in the lysate.

Fig. III.3b contains two bands of non-specific binding, but its band at 42 kDa is even more prominent than the band in Fig. III.4. This results shows that the HUC1-1 antibody from Santa Cruz Biotechnolgy bound very well to the actin.

Fig. III.3c contains only one band of non-specific binding and has a very low background. However, this HUC1-1 antibody from Thermo Scientific did not bind very well to the purified SMGA (control). For this reason, we did not use this antibody in further experiments.

From this data, we chose to use the HUC1-1 from MP Biomedical (Fig. III.3) to perform an immunoprecipitation. Upon a repeated trial of immunoprecipitation, we used the HUC1-1 from Santa Cruz Biotechnology.

Immunoprecipitation

After the Western blot detected actin in the lysate, we subsequently isolated the detected actin from the lysate by immunoprecipitation. Purified SMGA was also precipitated and run in parallel to the precipitated lysate to show that the precipitated band was actin. Diluted lysate was also run in parallel to these samples to confirm the actin band was also present in lysate, but at a lower concentration than in lane 4.

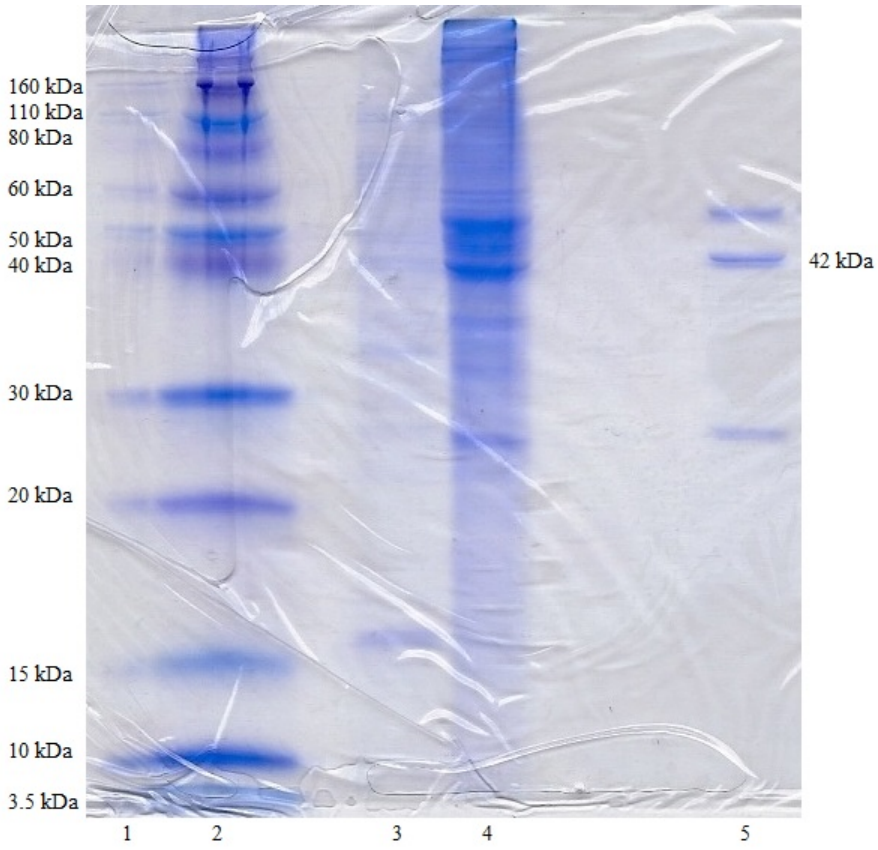


Fig. III.4.: Lane 1: Standard Marker, Lane 2: Standard Marker, Lane 3: Diluted Lysate, Lane 4: Precipitated Lysate, Lane 5: Precipitated SMGA.

Fig. III.4 shows an immunoprecipitation using HUC1-1 from MP Biomedical. The immunoprecipated band at 42 kDa in lane 4 was hypothesized to be actin.

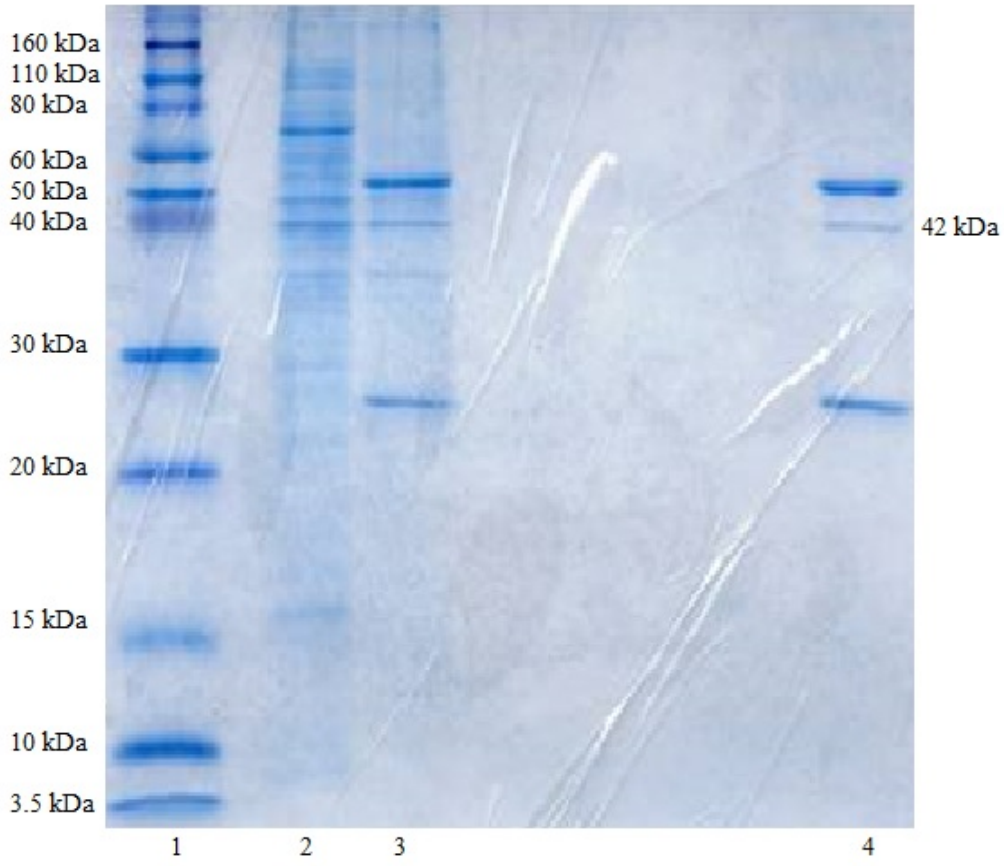


Fig. III.5.: Lane 1: Standard Marker, Lane 2: Diluted Lysate, Lane 3: Precipitated Lysate, Lane 4: Precipitated SMGA.

Fig. III.5 depicts an immunoprecipitation that was repeated in collaboration with Chevaun Johnson, a summer intern from Prairie View A&M University. We used HUC1-1 from Santa Cruz Biotechnology in order to obtain a more specific result.

Mass Spectrometry

Mass spectrometry analyzed the peptides in the immunoprecipitated band that was cut from the gel. Figures III.7 and III.9 show the sequence coverage of the analyzed peptides. Blank areas indicate that the computer did not analyze peptides at those areas. Yellow areas signify residues the computer analyzed and matched to its database. Green areas coordinate to residues the computer analyzed, but was unable to match to its database.

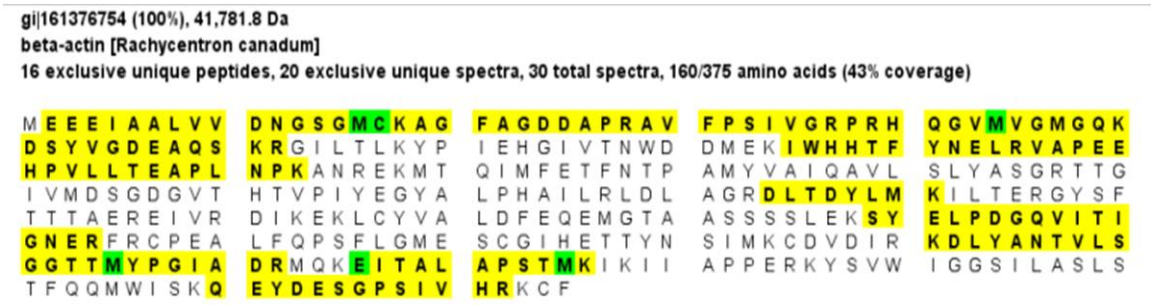


Fig. III.6.: Result from mass spectrometry shows the computer's unbiased match of the pulldown actin from the lysate to β-actin to Rachycentron canadum, a species of fish.

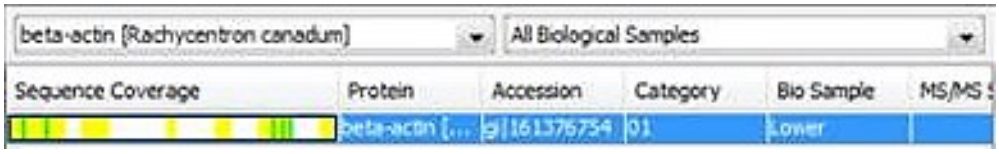


Fig. III.7.: Sequence: Lysate 1.

Figures III.6 and III.7 show the analysis of the band cut from the gel in Fig.III.4. Fig. III.6 shows a match to β-actin in Rachycentron canadum. The yellow areas in Fig. III.7 mean that most residues from the lysate's actin were detected at the N-terminus. However, the green lines signify that the computer was unable to match certain analyzed residues to its database, which suggests an alteration at the lysate's N-terminus. While the computer successfully matched the pulldown of our lysate to an actin isoform, we wanted to improve specificity and match our sample to SMGA.

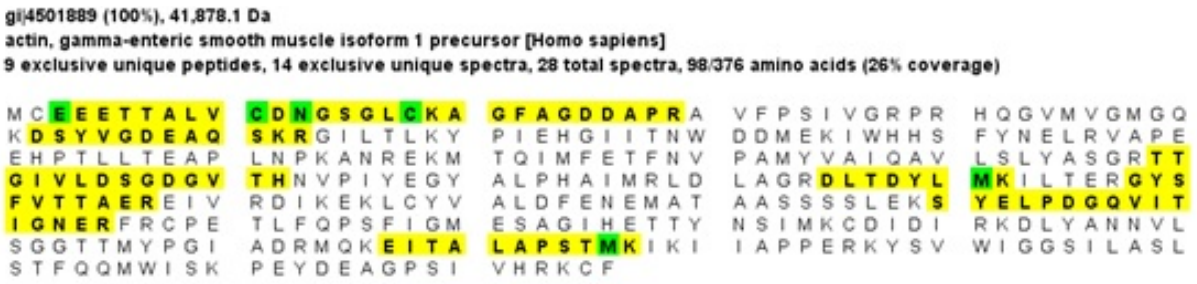


Fig. III.8.: The computer analyzed 26% of the immunoprecipitated sample and matched the sample to human SMGA with a 100% confidence level.

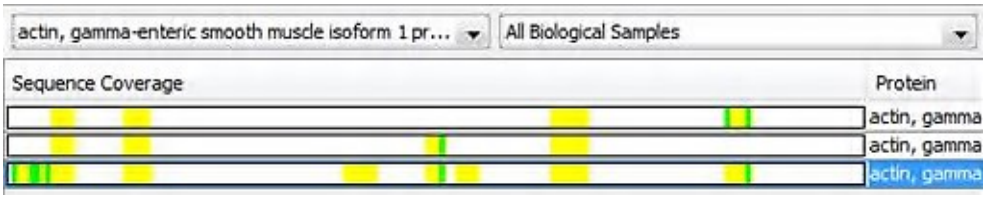


Fig. III.9.: Top sequence: Lysate 1, Middle sequence: Lysate 2, Bottom sequence: Purified SMGA.

The subsequent immunoprecipitation from Fig III.5 produced the results shown in Figures III.8 and III.9. These results matched our immunoprecipiated actin to human SMGA. Fig. III.9 shows that the computer failed to detect the N-terminus in the lysates (top and middle sequences). While green regions are visible in purified SMGA (the bottom sequence), the computer was still able to match most of the N-terminus in purified SMGA to human SMGA as demonstrated by the visible yellow regions. This result indicates that the N-terminus of SMGA from the lysates of prostate epithelial cells is unique as compared to purified SMGA from muscle cells.

CHAPTER IV

SUMMARY AND CONCLUSION

Previous studies have determined that prostate epithelial cells contain mRNA which encodes for SMGA [10]. The mRNA for SMGA has been detected and using anti-actin HUC1-1 antibody, it has been found that the expression of this protein hypothesized to be SMGA has been found to increase as the cancer becomes more aggressive.

The goal of this project was to isolate and determine that the actin in cancerous prostate epithelial cells was in fact SMGA. We were successfully able to support this hypothesis by culturing and lysing cancerous prostate epithelial cells and ultimately using the lysate to show that actin-specific antibody HUC1-1 was able to precipitate actin from the lysate. The precipitated actin was analyzed by mass spectroscopy and while initially matched to actin from a fish species, it subsequently matched to human actin, except at the N-terminus. This result shows that 1) immunoprecipitated actin from the prostate epithelial is identified as SMGA by mass spectrometry analysis 2) we can successfully purify prostate epithelial-specific SMGA through immunoprecipitation assays, 3) prostate epithelial-specific SMGA is unique from SMGA in smooth muscle cells at the N-terminus, where all actin isoforms vary.

It is possible that the N-terminus of prostate epithelial-specific SMGA was undetected either because it is not present or because there is an alteration at the N-terminus which is so distinct from SMGA in smooth muscle that the computer was unable to match any residues at that location of the sequence. Two explanations can be reasons for an alternation at the N-terminus: 1) alternate splicing of the gene, which would lead to a different mRNA sequence or 2) modification at the post-translational level. However, the past study conducted by Fillmore et. al. which determined that the complete mRNA sequence of this protein matched to SMGA discredits the possibility of gene splicing [10]. Therefore, the only possibility for this unique N-terminus in prostate epithelial-

specific SMGA is due to post translational modifications (PTM). Examples of chemical changes to the amino acid residues are ubiquitination, acetylation, and phosphorylation; each of which could be an explanation for what prevents the residues at the N-terminus from being read and led to a deletion or mistranslation into a unique N-terminus.

It is hoped that this study will stimulate further investigation in the field of cancer research. A further study for this project would be to sequence the N-terminus residues using Edman degradation and determine the difference between the sequenced prostate epithelial-specific SMGA from SMGA found in mature smooth muscle. In the long term, this sequence could be used to synthesize an antibody specific to this SMGA at its unique N-terminus to determine its role in the prostate epithelia.

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